PCT

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification [5] :	A1	(11) International Publication Number:	WO 95/21527
A01N 63/00, A61K 37/00, 49/00, G01N 1/00, 31/00, 33/15, 33/48		(43) International Publication Date:	17 August 1995 (17.08.95)

(21) International Application Number: PCT/US94/01616 (22) International Filing Date: 14 February 1994 (14.02.94) (71) Applicant *(for all designated States except US)*: THE GENERAL HOSPITAL CORPORATION [US/US]; 55 Fruit Street, Boston, MA 02114 (US). (72) Inventors; and (75) Inventors/Applicants *(for US only)*: SACHS, David, H. [US/US]; 77 Studio Road, Newton, MA 02166 (US). SYKES, Megan [US/US]; 197 Eighth Street #301, Charlestown, MA 02129-4230 (US). (74) Agents: MYERS, Paul, Louis et al.; Lahive & Cockfield, 60 State Street, Boston, MA 02109 (US).	(81) Designated States: AU, CA, JP, KP, KR, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). **Published** *With international search report.*

(54) Title: STEM CELL ENGRAFTMENT

(57) Abstract

A method of promoting the engraftment of exogenously administered hematopoietic stem cells in a recipient comprising administering myelosuppressive treatment to the recipient to create hematopoietic space, determining the white blood cell level of the recipient, administering the hematopoietic cells to the recipient during a period in which the white blood cell level is depressed.

STEM CELL ENGRAFTMENT

This application is a continuation in part of USSN 08/181,558, Sykes and Sachs, filed January 12, 1994, which is a continuation-in-part of USSN 08/129,608, filed September 29, 1993, which is a continuation-in-part of USSN 08/063,171, filed May 17, 1993, which is a continuation-in-part of USSN 07/797,555, filed November 22, 1991, and USSN 07/838,595, filed February 19, 1992, which is a continuation-in-part of USSN 07/817,761, filed August 1, 1993. This application is also a continuation-in-part of USSN 08/126,122, filed September 23, 1993, of USSN 08/150, 739, filed November 10, 1993, of USSN 08/177,655 Sykes and Sachs, filed January 5, 1994, which is a FWC of USSN 08/163,912. All of the above-recited United States Patent Applications are hereby incorporated by reference.

Background of the Invention

The invention relates to tissue and organ transplantation and to the engraftment of stem cells.

Summary of the Invention

The engraftment of exogenously supplied hematopoietic stem cells can be promoted by treating the recipient of the cells so as to induce hematopoietic space in the recipient. Hematopoietic space is commonly induced by radiation, but can also be induced by anti class I antibodies or by myelosuppressive drugs.

The inventors have discovered that myelosuppressive treatment sufficient to create hematopoietic space generally results in a reduction in white blood cell (WBC) levels (as revealed, e.g., by WBC counts) and that the WBC reduction serves as a marker for the presence of hematopoietic space. The marker is a conservative one since WBC counts may recover at a time when space is still present in an animal.

Accordingly, in one aspect, the invention features, a method of determining if a myelosuppressive or hematopoietic-space inducing treatment is sufficient to create hematopoietic space. The method includes administering a myelosuppressive treatment to a recipient, and determining the level of white blood cells in the recipient, e.g., by determining the WBC count of the recipient, a depression in the level of white blood cells being indicative of the presence or induction of hematopoietic space.

In preferred embodiments: the white blood cell level is compared to a white blood cell determination made prior to administration of myelosuppressive treatment; white blood cell levels are taken at two, three, four, or five time points after treatment; subsequent to determining the white blood cell level, hematopoietic stem

5   cells are administered to the recipient; the stem cells are administered during the period in which the white blood cell level is depressed.

In preferred embodiments: the recipient and the stem cell donor are from the same species; the recipient and the stem cell donor are from different, e.g., concordant or discordant, species; the recipient and the stem cell donor are from the

10   same species; the recipient is a mammal, (preferably a mammal other than a mouse), e.g., a primate, e.g., a human; the donor is a mammal, (preferably a mammal other than a mouse), e.g., a primate, e.g., a non-human primate, or a swine.

In another aspect, the invention features, a method of promoting the engraftment of exogenously administered hematopoietic stem cells in a recipient. The

15   method includes: administering myelosuppressive or hematopoietic-space-inducing treatment to the recipient, determining the white blood cell level of the recipient, administering the hematopoietic stem cells to the recipient (preferably the stem cells are administered during the period in which the white blood cell level is depressed), and, optionally, implanting in the recipient, a graft from a donor.

20   In preferred embodiments: the recipient and the stem cell donor are from the same species; the recipient and the stem cell donor are from different, e.g. concordant or discordant, species; the recipient and the stem cell donor are from the same species; the recipient is a mammal, (preferably a mammal other than a mouse), e.g., a primate, e.g., a human; the donor is a mammal, (preferably a mammal other

25   than a mouse), e.g., a primate, e.g., a non-human primate, or a swine.

In preferred embodiments: the graft is from the same individual which donates the stem cells; the graft is from an individual which is syngeneic with the individual which donates the stem cells; the graft is from an individual which is MHC matched with the individual which donates the stem cells.

30   In another aspect, the invention features a method of inducing tolerance in a recipient mammal (preferably a mammal other than a mouse), e.g., a primate, e.g., a human, of a first species, to a graft from a mammal (preferably a mammal other than a mouse), e.g., a swine, e.g., a miniature swine, of a second species, which graft expresses a major histocompatibility complex (MHC) antigen. The method includes

35   inserting DNA encoding an MHC antigen of the second species into a hematopoietic stem cell of the recipient mammal and allowing the MHC antigen encoding DNA to be expressed in the recipient.

In preferred embodiments: the method includes the step of, prior to administering the stem cells, administering a hematopoietic space inducing or

myelosuppressive treatment, e.g., irradiation, e.g., whole body irradiation, to the recipient, monitoring the white cell level of said recipient after the administration of the space inducing treatment, and administering the stem cells to the recipient while the white cell level is depressed.

5   Preferably, space for said hematopoietic stem cells is created by irradiating the recipient mammal with low dose whole body irradiation. Preferably, the low dose whole body irradiation is more than 100 rads and less than 400 rads. Alternatively, space can be created by administering to the recipient mammal anti-MHC class I antibodies or myelosuppressive drugs.

10   Preferably, levels of white blood cells are monitored by white blood cell counts. Preferably white blood cell counts in said recipient mammal are monitored for less than two weeks following creation of space within the recipient mammal. More preferably, white blood cell counts are monitored for less than one week.

           Another embodiment includes the further step of selecting a time for
15 administration of said hematopoietic stem cells when white blood cells levels are below normal in said recipient mammal. Preferably, the time for administering hematopoietic stem cells is within 4 or within 7 days following creation of space in the recipient mammal. Alternatively, hematopoietic stem cells can be administered within 14 or within 21 days following creation of space in the recipient mammal.

20   In preferred embodiments, the method includes administering to the recipient a short course of help reducing treatment, e.g., a short course of high dose cyclosporine treatment; the duration of the short course of help reducing treatment is approximately equal to or is less than the period required for mature T cells of the recipient species to initiate rejection of an antigen after first being stimulated by the
25 antigen; in more preferred embodiments the duration is approximately equal to or is less than two, three, four, five, or ten times the period required for a mature T cell of the recipient species to initiate rejection of an antigen after first being stimulated by the antigen. In other preferred embodiments the short course of help reducing treatment is administered in the absence of a treatment which stimulates the release of
30 a cytokine by mature T cells in the recipient, e.g., in the absence of Prednisone or similar compounds. In preferred embodiments: the help reducing treatment is begun before or at about the time the graft is introduced; the short course is perioperative; or the short course is postoperative.

           Preferred embodiments include those in which: the cell is removed from
35 the recipient mammal prior to the DNA insertion and returned to the recipient mammal after the DNA insertion; the DNA is obtained from the individual mammal from which the graft is obtained; the DNA is obtained from an individual mammal which is syngeneic with the individual mammal from which the graft is obtained; the DNA is obtained from an individual mammal which is MHC matched, and preferably

identical, with the individual mammal from which the graft is obtained; the DNA includes an MHC class I gene; the DNA includes an MHC class II gene; the DNA is inserted into the cell by transduction, e.g., by a retrovirus, e.g., by a Moloney-based retrovirus; and the DNA is expressed in bone marrow cells and/or peripheral blood

5   cells of the recipient for at least 14, preferably 30, more preferably 60, and most preferably 120 days, after the DNA is introduced into the recipient.

In another aspect, the invention features a method of inducing tolerance in a recipient mammal, (preferably a mammal other than a mouse), e.g., a primate, e.g., a human, to a graft obtained from a donor of the same species, which graft

10   expresses an MHC antigen. The method includes: inserting DNA encoding an MHC antigen of the donor into a bone marrow hematopoietic stem cell of the recipient; allowing the MHC antigen encoding DNA to be expressed in the recipient.

In preferred embodiments: the method includes the step of, prior to administering the stem cells, administering a hematopoietic space inducing or

15   myelosuppressive treatment, e.g., irradiation, e.g., whole body irradiation, to the recipient, monitoring the white cell level of said recipient after the administration of the space inducing treatment, and administering the stem cells to the recipient while the white cell level is depressed.

Preferably, space for said hematopoietic stem cells is created by

20   irradiating the recipient mammal with low dose whole body irradiation. Preferably, the low dose whole body irradiation is more than 100 rads and less than 400 rads. Alternatively, space can be created by administering to the recipient mammal anti-MHC class I antibodies or myelosuppressive drugs.

Preferably, levels of white blood cells are monitored by white blood cell

25   counts. Preferably white blood cell counts in said recipient mammal are monitored for less than two weeks following creation of space within the recipient mammal. More preferably, white blood cell counts are monitored for less than one week.

Another embodiment includes the further step of selecting a time for administration of said hematopoietic stem cells when white blood cells levels are

30   below normal in said recipient mammal. Preferably, the time for administering hematopoietic stem cells is within 4 or within 7 days following creation of space in the recipient mammal. Alternatively, hematopoietic stem cells can be administered within 14 or within 21 days following creation of space in the recipient mammal.

In preferred embodiments: the method further includes administering to

35   the recipient a short course of help reducing treatment, e.g., a short course of high dose cyclosporine: the duration of the short course of help reducing treatment is approximately equal to or is less than the period required for mature T cells of the recipient species to initiate rejection of an antigen after first being stimulated by the antigen; in more preferred embodiments the duration is approximately equal to or is

less than two, three, four, five, or ten times the period required for a mature T cell of the recipient species to initiate rejection of an antigen after first being stimulated by the antigen. In other preferred embodiments the short course of help reducing treatment is administered in the absence of a treatment which stimulates the release of a cytokine by mature T cells in the recipient, e.g., in the absence of Prednisone or similar compounds. In preferred embodiments: the help reducing treatment is begun before or at about the time the graft is introduced; the short course is perioperative, the short course is postoperative; or the donor and recipient are class I matched.

Preferred embodiments include those in which: the cell is removed from the recipient prior to the DNA insertion and returned to the recipient after the DNA insertion; the DNA includes a MHC class I gene; the DNA includes a MHC class II gene; the DNA is inserted into the cell by transduction, e.g. by a retrovirus, e.g., by a Moloney-based retrovirus; and the DNA is expressed in bone marrow cells and/or peripheral blood cells of the recipient at least 14, preferably 30, more preferably 60, and most preferably 120 days, after the DNA is introduced into the recipient.

In another aspect, the invention features a method of inducing tolerance in a recipient mammal of a first species, (preferably a mammal other than a mouse) e.g., a primate, e.g., a human, to a graft obtained from a mammal of a second, preferably a concordant or discordant species, e.g., a swine, e.g., a miniature swine, or a discordant primate species. The method includes: prior to or simultaneous with transplantation of the graft, introducing, e.g., by intravenous injection, into the recipient mammal hematopoietic stem cells, e.g., bone marrow cells or fetal liver or spleen cells, of the second species (preferably the hematopoietic stem cells home to a site in the recipient mammal); and (optionally) inactivating the natural killer cells of said recipient mammal, e.g., by prior to introducing the hematopoietic stem cells into the recipient mammal, introducing into the recipient mammal an antibody capable of binding to natural killer cells of said recipient mammal.

In preferred embodiments: the method includes the step of, prior to administering the stem cells, administering a hematopoietic space inducing or myelosuppressive treatment, e.g., irradiation, e.g., whole body irradiation, to the recipient, monitoring the white cell level of said recipient after the administration of the space inducing treatment, and administering the stem cells to the recipient while the white cell level is depressed.

Preferably, space for said hematopoietic stem cells is created by irradiating the recipient mammal with low dose whole body irradiation. Preferably, the low dose whole body irradiation is more than 100 rads and less than 400 rads. Alternatively, space can be created by administering to the recipient mammal anti-MHC class I antibodies or myelosuppressive drugs.

Preferably, levels of white blood cells are monitored by white blood cell counts. Preferably white blood cell counts in said recipient mammal are monitored for less than two weeks following creation of space within the recipient mammal. More preferably, white blood cell counts are monitored for less than one week.

5   Another embodiment includes the further step of selecting a time for administration of said hematopoietic stem cells when white blood cells levels are below normal in said recipient mammal. Preferably, the time for administering hematopoietic stem cells is within 4 or within 7 days following creation of space in the recipient mammal. Alternatively, hematopoietic stem cells can be administered within

10   14 or within 21 days following creation of space in the recipient mammal.

In preferred embodiments: the method includes administering to the recipient a short course of help reducing treatment, e.g., a short course of high dose cyclosporine; the duration of the short course of help reducing treatment is approximately equal to or is less than the period required for mature T cells of the

15   recipient species to initiate rejection of an antigen after first being stimulated by the antigen; in more preferred embodiments the duration is approximately equal to or is less than two, three, four, five, or ten times, the period required for a mature T cell of the recipient species to initiate rejection of an antigen after first being stimulated by the antigen. In other preferred embodiments the short course of help reducing

20   treatment is administered in the absence of a treatment which stimulates the release of a cytokine by mature T cells in the recipient, e.g., in the absence of Prednisone or similar compounds. In preferred embodiments: the help reducing treatment is begun before or at about the time the graft is introduced; or the short course is perioperative, the short course is postoperative.

25   As will be explained in more detail below, the hematopoietic cells prepare the recipient for the graft that follows, by inducing tolerance at both the B-cell and T-cell levels. Preferably, hematopoietic cells are fetal liver or spleen, or bone marrow cells, including immature cells (i.e., undifferentiated hematopoietic stem cells; these desired cells can be separated out of the bone marrow prior to administration), or

30   a complex bone marrow sample including such cells can be used.

One source of anti-NK antibody is anti-human thymocyte polyclonal anti-serum. As is discussed below preferably, a second, anti-mature T cell antibody can be administered as well, which lyses T cells as well as NK cells. Lysing T cells is advantageous for both bone marrow and xenograft survival. Anti-T cell antibodies are

35   present, along with anti-NK antibodies, in anti-thymocyte anti-serum. Repeated doses of anti-NK or anti-T cell antibody may be preferable. Monoclonal preparations can be used in the methods of the invention.

Other preferred embodiments include: the step of introducing into the recipient mammal, donor species-specific stromal tissue, preferably hematopoietic

stromal tissue, e.g., fetal liver or thymus. In preferred embodiments: the stromal tissue is introduced simultaneously with, or prior to, the hematopoietic stem cells; the hematopoietic stem cells are introduced simultaneously with, or prior to, the antibody.

Other preferred embodiments include those in which: the same mammal of the second species is the donor of one or both the graft and the hematopoietic cells; and the antibody is an anti-human thymocyte polyclonal anti-serum, obtained, e.g., from a horse or pig.

Other preferred embodiments include: the step of prior to hematopoietic stem cell transplantation, irradiating the recipient mammal with low dose, e.g., between about 100 and 400 rads, whole body irradiation to deplete or partially deplete the bone marrow of said recipient; and the step of prior to hematopoietic stem cell transplantation, inactivating T cells in the thymus, by, e.g., administration of anti-T cell antibodies or by irradiating the recipient mammal with, e.g., about 700 rads of thymic irradiation.

Other preferred embodiments include: the step of prior to hematopoietic stem cell transplantation, depleting natural antibodies from the blood of the recipient mammal, e.g., by hemoperfusing an organ, e.g., a liver or a kidney, obtained from a mammal of the second species. (In organ hemoperfusion antibodies in the blood bind to antigens on the cell surfaces of the organ and are thus removed from the blood.)

In other preferred embodiments: the method further includes, prior to hematopoietic stem cell transplantation, introducing into the recipient an antibody capable of binding to mature T cells of said recipient mammal.

Other preferred embodiments further include the step of introducing into the recipient a graft obtained from the donor, e.g., a graft which is obtained from a different organ than the hematopoietic stem cells, e.g., a liver or a kidney.

In another aspect, the invention features a method of inducing tolerance in a recipient mammal, (preferably a mammal other than a mouse) e.g., a primate, e.g., a human, to a graft obtained from a donor, e.g., of the same species. The method includes: prior to or simultaneous with transplantation of the graft, introducing, e.g., by intravenous injection, into the recipient hematopoietic stem cells, e.g. bone marrow cells or fetal liver or spleen cells, of a mammal, preferably the donor (preferably the hematopoietic stem cells home to a site in the recipient); and (optionally) inactivating the natural killer cells or T cells (or both) of the recipient, e.g., by prior to introducing the hematopoietic stem cells into the recipient, introducing into the recipient an antibody capable of binding to natural killer cells or T cells (or both) of the recipient.

In preferred embodiments: the method includes the step of, prior to administering the stem cells, administering a hematopoietic space inducing or myelosuppressive treatment, e.g., irradiation, e.g., whole body irradiation, to the recipient, monitoring the white cell level of said recipient after the administration of

the space inducing treatment, and administering the stem cells to the recipient while
the white cell level is depressed.

Preferably, space for said hematopoietic stem cells is created by
irradiating the recipient mammal with low dose whole body irradiation. Preferably,
5   the low dose whole body irradiation is more than 100 rads and less than 400 rads.
Alternatively, space can be created by administering to the recipient mammal anti-
MHC class I antibodies or myelosuppressive drugs.

Preferably, levels of white blood cells are monitored by white blood cell
counts. Preferably white blood cell counts in said recipient mammal are monitored for
10 less than two weeks following creation of space within the recipient mammal. More
preferably, white blood cell counts are monitored for less than one week.

Another embodiment includes the further step of selecting a time for
administration of said hematopoietic stem cells when white blood cells levels are
below normal in said recipient mammal. Preferably, the time for administering
15 hematopoietic stem cells is within 4 or within 7 days following creation of space in the
recipient mammal. Alternatively, hematopoietic stem cells can be administered within
14 or within 21 days following creation of space in the recipient mammal.

In preferred embodiments: the method includes administering to the
recipient a short course of help reducing treatment, e.g., a short course of high dose
20 cyclosporine: the duration of the short course of help reducing treatment is
approximately equal to or is less than the period required for mature T cells of the
recipient species to initiate rejection of an antigen after first being stimulated by the
antigen; in more preferred embodiments the cluration is approximately equal to or is
less than two, three, four, five, or ten times the period required for a mature T cell of
25 the recipient species to initiate re~ection of an antigen after first being stimulated by
the antigen. In other preferred embodiments the short course of help reducing
treatment is administered in the absence of a treatment which stimulates the release of
a cytokine by mature T cells in the recipient, e.g., in the absence of Prednisone or
similar compounds. In preferred embodiments: the help reducing treatment is begun
30 before or at about the time the graft is introduced; the short course is perioperative, the
short course is postoperative; the donor and recipient are class I matched.

In preferred embodiments the hematopoietic stem cells are introduced
simultaneously with, or prior to administration of the antibody; the antibody is an anti-
human thymocyte polyclonal anti-serum; and the anti-serum is obtained from a horse
35 or pig.

Other preferred embodiments include: the further step of, prior to
hematopoietic stem cell transplantation, introducing into the recipient mammal an
antibody capable of binding to mature T cells of the recipient mammal; and those in
which the same individual is the donor of both the graft and the bone marrow.

SUBSTITUTE SHEET (RULE 26)

Other preferred embodiments include: further including the step of prior to hematopoietic stem cell transplantation, irradiating the recipient with low dose, e.g., between about 100 and 400 rads, whole body irradiation to completely or partially deplete the bone marrow of the recipient; and further including the step of prior to

5   hematopoietic stem cell transplantation, inactivating T cells in the thymus, by, e.g., administration of anti-T cell antibodies or by irradiating the recipient with, e.g., about 700 rads of, thymic irradiation.

Other preferred embodiments include: the further step prior of to bone marrow transplantation, absorbing natural antibodies from the blood of the recipient

10   by hemoperfusing an organ, e.g., the liver, or a kidney, obtained from the donor.

Preferred embodiments include: the step of introducing into the recipient mammal, donor speciesspecific stromal tissue, preferably hematopoietic stromal tissue, e.g., fetal liver or thymus.

Other preferred embodiments further include the step of introducing into

15   the recipient, a graft obtained from the donor, e.g., a graft which is obtained from a different organ than the hematopoietic stem cells, e.g., a liver or a kidney.

The invention further provides a method for reducing or eliminating immature or mature thymic T cells without thymic irradiation in a recipient mammal comprising administering to the recipient mammal at least one T cell ablative or T

20   cell-depleting antibody at a dose sufficient to reduce or eliminate immature or mature thymic T cells. Preferably, T cell ablative antibodies are administered to the recipient prior to administering an allograft to the recipient. Preferred T cell ablative antibodies are anti-CD4 and anti-CD8 antibodies. Preferably, the anti-CD4 and anti-CD8 antibodies are coadministered to the recipient.

25   "Help reduction", as used herein, means the reduction of T cell help by the inhibition of the release of at least one cytokine, e.g. , any of IL-2, IL-4, IL-6, gamma interferon, or TNF, from T cells of the recipient at the time of the first exposure to an antigen to which tolerance is desired. The inhibition induced in a recipient's T cell secretion of a cytokine must be sufficient such that the recipient is

30   tolerized to an antigen which is administered during the reduction of help. Although not being bound by theory, it is believed that the level of reduction is one which substantially eliminates the initial burst of IL-2 which accompanies the first recognition of a foreign antigen but which does not eliminate all mature T cells, which cells may be important in educating and producing tolerance.

35   "A help reducing agent", as used herein, is an agent, e.g., an immunosuppressive drug, which results in the reduction of cytokine release. Examples of help reducing agents are cyclosporine, FK-506, and rapamycin. Anti-T cell antibodies, because they can eliminate T cells, are not preferred for use as help reducing agents. A help reducing agent must be administered in sufficient dose to give

the level of inhibition of cytokine release which will result in tolerance. The help reducing agent should be administered in the absence of treatments which promote cytokine, e.g., IL-2, release. Putative agents help reducing agents can be prescreened by *in vitro* or *in vivo* tests, e.g., by contacting the putative agent with T cells and determining the ability of the treated T cells to release a cytokine, e.g., IL-2. The inhibition of cytokine release is indicative of the putative agent's efficacy as a help reducing agent. Such prescreened putative agents can then be further tested in a kidney transplant assay. In a kidney transplant assay a putative help reducing agent is tested for efficacy by administering the putative agent to a recipient monkey and then implanting a kidney from a Class II matched Class I and minor antigen mismatched donor monkey into the recipient. Tolerance to the donor kidney (as indicated by prolonged acceptance of the graft) is indicative that the putative agent is, at the dosage tested, a help reducing agent.

"Short course of a help reducing agent", as used herein, means a transitory non-chronic course of treatment. The treatment should begin before or at about the time of transplantation of the graft. Alternatively, the treatment can begin before or at about the time of the recipient's first exposure to donor antigens. Optimally, the treatment lasts for a time which is approximately equal to or less than the period required for mature T cells of the recipient species to initiate rejection of an antigen after first being stimulated by the antigen. The duration of the treatment can be extended to a time approximately equal to or less than two, three, four, five, or ten times, the period required for a mature T cell of the recipient species to initiate rejection of an antigen after first being stimulated by the antigen. The duration will usually be at least equal to the time required for mature T cells of the recipient species to initiate rejection of an antigen after first being stimulated by the antigen. In pigs and monkeys, about 12 days of treatment is sufficient. Experiments with cyclosporine A (10 mg/kg) in pigs show that 6 days is not sufficient. Other experiments in monkeys show that IL-2 administered on day 8, 9, or 10 of cyclosporine A treatment will result in rejection of the transplanted tissue. Thus, 8, 9, or 10 days is probably not sufficient in pigs. In monkeys, a dose of 10mg/kg cyclosporine with a blood level of about 500-1,000 ng/ml is sufficient to induce tolerance to Class II matched Class I and minor antigen mismatched kidneys. The same blood level, 500-1,000 ng/ml, is sufficient to induce tolerance in pigs. Long-term administration of 5mg/kg prevents rejection (by long term immune suppression) but does not result in tolerance.

"Tolerance", as used herein, refers to the inhibition of a graft recipient's immune response which would otherwise occur, e.g., in response to the introduction of a nonself MHC antigen into the recipient. Tolerance can involve humoral, cellular, or both humoral and cellular responses. The term tolerance refers to states characterized by total or partial inhibition of the immune response.

"Hematopoietic stem cell", as used herein, refers to a cell which is capable of developing into a mature myeloid and/or lymphoid cell. Stem cells derived from the cord blood of the recipient or the donor can be used in methods of the invention. See U.S. Patent 5,192,553, hereby incorporated by reference, and U.S. Patent 5,004,681, hereby incorporated by reference. Other sources of stem cells are bone marrow, fetal liver, fetal spleen, neonatal liver, and neonatal spleen.

"MHC antigen", as used herein, refers to a protein product of one or more MHC genes; the term includes fragments or analogs of products of MHC genes which can evoke an immune response in a recipient organism. Examples of MHC antigens include the products (and fragments or analogs thereof) of the human MHC genes, i.e., the HLA genes. MHC antigens in swine, e.g., miniature swine, include the products (and fragments and analogs thereof) of the SLA genes, e.g., the DRB gene.

"Miniature swine", as used herein, refers to wholly or partially inbred animal.

"Graft", as used herein, refers to a body part, organ, tissue, or cells. Grafts may consist of organs such as liver, kidney, heart or lung; body parts such as bone or skeletal matrix; tissue such as skin, intestines, endocrine glands; or progenitor stem cells of various types.

"A discordant species combination", as used herein, refers to two species in which hyperacute rejection occurs when a graft is grafted from one to the other. Generally, discordant species are from different orders, while non-discordant species are from the same order. For example, rats and mice are non-discordant species, i.e. their MHC antigens are substantially similar, and they are members of the same order, rodentia.

"Stromal tissue", as used herein, refers to the supporting tissue or matrix of an organ, as distinguished from its functional elements or parenchyma. Sources include adult, fetal, or neonatal thymus or bone marrow.

"Hematopoietic space" as used herein, refers to the capacity of a mammal to accept engraftment of exogenously supplied hematopoietic stem cells. Hematopoetic space is an induced condition and it exists when a treatment renders the recipient more receptive to engraftment of exogenously administered hematopoietic stem cells.

"Myelosuppressive treatment" as used herein, refers to any treatment which renders a recipient more susceptible to engraftment of exogenously administered stem cells or which kills or inhibits the maintenance, growth, or proliferation of stem cells. Irradiation, antibodies, or drugs or chemicals can be used as myelosuppressive agents.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

<u>Detailed Description</u>

The drawings will first be briefly described.

<u>Drawings</u>

5   Figure 1A is a graph depicting the number of white blood cells in the peripheral blood of irradiated mice on days following irradiation. Cell counts are from the following mice: (•) Untreated B6; (■) Untreated B6.Ly-5.2; (·····) B6 mice injected with 1.5×10^7 B6.Ly-5.2 BMC; (_··_) B6 mice irradiated with 0.5 Gy and injected with 1.5×10^7 B6.Ly-5.2 BMC;

(___) B6 mice irradiated with 1.5 Gy alone; (----) B6 mice irradiated with 1.5 Gy and injected with 1.5×10^7 B6.Ly-5.2 BMC; (— —) B6 mice irradiated with 3 Gy and injected with 1.5×10^7 B6.Ly-5.2 BMC.

Figure 1B is a graph depicting hemoglobin levels in the peripheral blood of irradiated mice on days following irradiation. Mice are as described in Figure 1A.

Figure 1C is a graph depicting platelet counts in the peripheral blood of irradiated mice on days following irradiation. Mice are as described in Figure 1A.

<u>Overview</u>

The invention provides several methods of inducing tolerance to foreign antigens, e.g., to antigens on allogeneic or xenogeneic tissue or organ grafts. These methods can be used individually or in combination.

<u>A depression in WBC corresponds to a window for stem cell engraftment</u>

It has been found that myelosuppressive treatment, e.g., whole body irradiation (WBI), administered prior to hematopoietic stem cell transplantation is generally necessary to create "space" for the donor hematopoietic stem cells. It is important for the promotion of engraftment rather than merely serving an immunsuppressive function. It further has been discovered that the space created by WBI of a recipient persists for a period of time following irradiation and that hematopoietic stem cells can be successfully transplanted within this "window" of time when space for donor hematopoietic stem cells persists in the recipient. It further has been discovered that the level of white cells in the periphery (as revealed in white blood cell (WBC) counts) can be used as an indicator of the presence of space for donor hematopoietic stem cells in the recipient: white blood cells counts decrease from normal levels following WBI of the recipient during the permissive period when bone marrow engraftment can be successfully accomplished. White blood cells counts in the recipient gradually increase back to normal levels following WBI, although the window for bone marrow engraftment can persists for a period of time after WBC counts have returned to normal in the recipient.

To diminish toxicity to the recipient, a minimal dose of WBI which can still prepare the recipient for bone marrow engraftment is used. A preferred low dose of irradiation is between about 100 to 400 rads WBI. Successful bone marrow
5   engraftment can be achieved if bone marrow transplantation is performed on the same day as WBI (day 0). It has now been discovered that high level, long term (i.e., stable) donor-type repopulation can also be achieved if bone marrow transplantation is performed as many as about seven days following WBI (i.e., bone marrow transplantation can be performed between day 0 and day 7 following WBI).
10   Somewhat less successful repopulation (i.e., mixed chimerism) is achieved if bone marrow transplantation is performed up to 14 days following WBI. Somewhat less successful repopulation (i.e., mixed chimerism) is also achieved if bone marrow transplantation is performed up to 21 days following WBI.

15   White blood cell counts in the recipient can be used as an indicator of the permissive period for bone marrow engraftment following WBI. It has been found that WBC counts in the recipient decline between about day 2 and day 7 following WBI at a dose sufficient to allow engraftment, whereas WBI at a lower dose does not result in decreased WBC counts following irradiation or successful engraftment.
20   Accordingly, WBC counts can serve as a marker for the presence of space for HSC engraftment and can be monitored to monitor space for HSC engraftment in the recipient. Preferably, WBC counts are monitored within about two weeks following irradiation, since counts in the recipient return to normal levels by about day 14 following irradiation. Although WBC counts return to normal by about 14 days
25   following irradiation, some degree of engraftment can still be achieved as many as about 21 days following irradiation. As stated above, engraftment can generally be achieved at just after irradiation. It may, however, be desirable to delay administration of stem cells.

30   WBC counts can be monitored by obtaining a blood sample from the recipient and counting WBCs within the sample by standard techniques. WBCs can be counted manually (e.g., with a hemocytometer) or more preferably are counted using an automated cell counter (e.g., System 9000; Serono-Baker Diagnostics Inc., Allentown, PA). WBC counts can be compared to a known standard to determine
35   whether the WBC count at a particular time following WBI (or other suitable procedure to create space) is normal, below normal or above normal. Normal WBC counts for different species are known in the art. Additionally, a WBC count for the recipient can be determined prior to WBI, and if necessary prior to other treatments of the recipient which could affect WBC counts (e.g., administration of ablative

antibodies), and this value can be used as a normal WBC count standard against which other WBC counts can be compared.

Induction of hematopoietic space

Procedures alternative to WBI can be used to create "space" for donor b hematopoietic stem cells within a recipient. For example, space can be created by treating the recipient with a monoclonal antibody against MHC class I antigens expressed by the recipient (see e.g., Voralia, M. et al. (1987) Transplantation 44:487) or space can be created by treating the recipient with myelosuppressive drugs (see e.g., Lapidot, T. et al. (1990) Proc. Natl. Acad. Sci. USA 87:4595). As for WBI, space created within the recipient for bone marrow transplantation by other mechanisms (e.g., anti-MHC class I treatment or myelosuppressive drugs) can be assessed by monitoring WBC counts in the recipient.

Elimination of thymocytes

It has further been discovered that thymic irradiation of a recipient (e.g., with about 700 rads) can be replaced by additional treatment of the recipient with T cell ablative antibodies. Accordingly, in another embodiment, the method of the invention further comprises introducing into the recipient mammal at least one antibody capable of binding to mature or immature T cells of the recipient mammal at a dose sufficient to induce tolerance to a graft without thymic irradiation of the recipient mammal. The preferred T cell ablative antibody treatment procedure comprises the combined use of antibodies directed against CD4 and CD8 molecules on the surface of T cells (i.e., combined administration of an anti-CD4 antibody and an anti-CD8 antibody). An appropriate dose of T cell ablative antibodies to be used instead of thymic irradiation can be determined by treating a recipient mammal with increasing dosages of one or more T cell ablative antibodies (e.g., an anti-CD4 antibody and an anti-CD8 antibody used in combination) in the absence of thymic irradiation and determining the dose of antibody needed to induce tolerance to an allograft, using transplantation procedures disclosed in the Examples.

Detailed Protocol

In the following protocol for preparing a cynomolgus monkey for receipt of a kidney from a miniature swine donor, zero time is defined as the moment that the arterial and venous cannulas of the recipient are connected to the liver to be perfused (note zero time is chosen to fall within the window of white blood cell level, as described herein).

On day -1 a commercial preparation (Upjohn) of horse anti-human anti-thymocyte globulin (ATG) is injected into the recipient. ATG eliminates mature T cells and natural killer cells that would otherwise cause rejection of the bone marrow cells used to induce tolerance. The recipient is anesthetized, an IV catheter is inserted

5 into the recipient, and 6 ml of heparinized whole blood are removed before injection. The ATG preparation is then injected (50 mg/kg) intravenously. Six ml samples of heparinized whole blood are drawn for testing at time points of 30 min., 24 hrs and 48 hrs. Blood samples are analyzed for the effect of antibody treatment on natural killer cell activity (testing on K562 targets) and by FACS analysis for lymphocyte

10 subpopulations, including CD4, CD8, CD3, CDllb, and CD16. Preliminary dat~ from both assays indicate that both groups of cells are eliminated by the administration of ATG. If mature T cells and NK cells are not eliminated, ATG can be re-administered at later times in the procedure, both before and after organ transplantation.

Sublethal irradiation is administered to the recipient between days -1 and

15 -8. Irradiation is necessary to eliminate enough of the recipient's endogenous BMC to stimulate hematopoiesis of the newly introduced foreign BMC. Sublethal total body irradiation is sufficient to permit engraftment with minimal toxic effects to the recipient. Whole body radiation (150 Rads) was administered to cynomolgus monkey recipients from a bilateral (TRBC) cobalt teletherapy unit at 10 Rads/min. Subsequent

20 to the administration of the first dose of irradiation the recipients white blood cell count is determined on a daily basis. A depression in the count corresponds to the onset of the window for stem cell engraftment. Local irradiation of the thymus (700 Rads) was also employed in order to facilitate engraftment. This can be replaced with administeration of anti T cell antibodies .

25 Natural antibodies are a primary cause of organ rejection. To remove natural antibodies from the recipient's circulation prior to transplantation, on day 0 an operative absorption of natural antibodies (nAB) is performed, using a miniature swine liver, as follows. At -90 minutes the swine donor is anesthetized, and the liver prepared for removal by standard operative procedures. At -60 minutes the recipient

30 monkey is anesthetized. A peripheral IV catheter is inserted, and a 6 ml sample of whole blood is drawn. Through mid-line incision, the abdominal aorta and the vena cava are isolated. Silastic cannulas containing side ports for blood sampling are inserted into the blood vessels.

At -30 minutes the liver is perfused in situ until it turns pale, and then

35 removed from the swine donor and placed into cold Ringers Lactate. The liver is kept cold until just prior to reperfusion in the monkey. A liver biopsy is taken. At -10 minutes the liver is perfused with warm albumin solution until the liver is warm (37 degrees).

At 0 time the arterial and venous cannulas of the recipient are connected to the portal vein and vena cava of the donor liver and perfusion is begun. Liver biopsies are taken at 30 minutes and 60 minutes, respectively. Samples of recipient blood are also drawn for serum at 30 minutes and 60 minutes respectively. At 60

5   minutes the liver is disconnected from the cannulas and the recipient's large blood vessels are repaired. The liver, having served its function of absorbing harmful natural antibodies from the recipient monkey, is discarded. Additional blood samples for serum are drawn from the recipient at 2, 24, and 48 i hours. When this procedure was performed on two sequential perfusions of swine livers, the second liver showed no

10   evidence of mild ischemic changes during perfusion. At the end of a 30 minute perfusion the second liver looked grossly normal and appeared to be functioning, as evidenced by a darkening of the venous o~tflow blood compared to the arterial inflow blood in the two adjacent cannulas. Tissue sections from the livers were normal, but immunofluorescent stains showed IgM on endothelial cells. Serum samples showed a

15   decrease in natural antibodies.

To promote long-term survival of the implanted organ through T-cell and B-cell mediated tolerance, donor bone marrow cells are administered to the recipient to form chimeric bone marrow. The presence of donor antigens in the bone marrow allows newly developing B cells, and newly sensitized T cells, to recognize

20   antigens of the donor as self, and thereby induces tolerance for the implanted organ from the donor. To stabilize the donor BMC, donor stromal tissue, in the form of tissue slices of fetal liver, thymus, and/or fetal spleen are transplanted under the kidney capsule of the recipient. Stromal tissue is preferably implanted simultaneously with, or prior to, administration of hematopoietic stem cells, e.g., BMC, or a fetal liver

25   cell suspension.

To follow chimerism, two color flow cytometry can be used. This assay uses monoclonal antibodies to distinguish between donor class I major histocompatibility antigens and leukocyte common antigens versus recipient class I major histocompatibility antigens.

30   BMC can in turn be injected either simultaneously with, or preceding, organ transplant. Bone marrow is harvested and injected intravenously (7.5×10^8/kg) as previously described (Pennington et al., 1988, transplantation 45:21-26). Should natural antibodies be found to recur before tolerance is induced, and should these antibodies cause damage to the graft, the protocol can be modified to permit sufficient

35   time following BMT for humoral tolerance to be established prior to organ grafting.

The approaches described above are designed to synerglst~cally prevent the problem of transplant rejection. When a kidney is implanted into a cynomolgus monkey following liver absorption of natural antibodies, without use of bone marrow transplantation to induce tolerance, renal functions continued for 1-2 days before

rejection of the kidney. When foru steps of the procedure were performed (absorption of natural antibodies by liver perfusion, administration of ATG, sublethal irradiation and bone marrow infusion, followed by implant of a procine kidney into a primate recipient), the kidney survived 7 days before rejection. Despite rejection of the

5   transplanted organ, the recipient remained healthy.

When swine fetal liver and thymic stromal tissue were implanted under the kidney capsule of two sublethally irradiated SCID mice, 25-50% of peripheral blood leukocytes were of donor lineage two weeks post-transplantation. A significant degree of chimerism was not detected in a third animal receiving feral liver without

10   thymus.

The methods of the invention may be employed in combination, as described, or in part.

The method of introducing bone marrow cells may be altered, particularly by (1) increasing the time interval between injecting hematopoietic stem

15   cells and implanting the graft; (2) increasing or decreasing the amount of hematopoietic stem cells injected; (3) varying the number of hematopoietic stem cell injections; (4) varying the method of delivery of hematopoietic stem cells; (5) varying the tissue source of hematopoietic stem cells, e.g., a fetal liver cell suspension may be used; of (6) varying the donor source of hematopoeitic stem cells. Although

20   hematopoietic stem cells derived from the graft donor are preferable, hematopoietic stem cells may be obtained from other individuals or species, or from genetically-engineered inbred donor strains, or from *in vitro* cell culture.

Methods of preparing the recipient for transplant of hematopoeitic stem cells Amy be varied. For instance, the recipient may undergo a splenectomy or a

25   thymectomy. The latter would preferably by administered prior to the non-myeloablative regimen, e.g., at day -14.

Hemoperfusion of natural antibodies may: (1) make use of other vascular organs, e.g., liver, kidney, intestines; (2) make use of multiple sequential organs; (3) vary the length of time each organ is perfused; (4) vary the donor of the

30   perfused organ. Irradiation of the recipient may make use of: (1) varying the absorbed dose of whole body radiation below the sublethal range; (2) targeting different body parts (e.g., thymus, spleen); (3) varying the rate of irradiation (e.g., 10 rads/min, 15 rads/min); or (4) varying the time interval between irradiation and transplant of hematopoeitic stem cells; any time interval between 1 and 14 days can be

35   used, and certain advantages may flow from use of a time interval of 4-7 days. Antibodies introduced prior to hematopoietic cell transplant may be varied by: (1) using monoclonal antibodies to T cell subsets or NK cells (e.g., anti-NKH1$_A$, as described by United States Patent No. 4,772,552 to Hercend, et al., hereby incorporated by reference); (2) preparing anti-human ATB in other mammalian hosts

(e.g., monkey, pig, rabbit, dog); or (3) using anti-monkey ATG prepared in any of the above mentioned hosts.

The methods of the invention may be employed with other mammalian recipients (e.g., rhesus monkeys) and may use other mammalian donors (e.g.,

5   primates, sheep, or dogs).

As an alternative or adjunct to hemoperfusion, host antibodies can be depleted by administration of an excess of hematopoietic cells.

EXAMPLE

10

The need for whole body irradiation (WBI) as a preparative step for allogeneic bone marrow transplantation could reflect a need to produce "space" within the recipient in order to achieve hematopoietic stem cell (HSC) engraftment or could reflect the need for the immunosuppressive effect of WBI. To determine whether

15   WBI is needed to create "space" or is needed only for its immunsuppressive effects in allogeneic transplant conditions, a syngeneic transplant system was used. In this example, an Ly-5 congenic strain combination was used involving B6.Ly-5.2 (Ly-5.1) donors and C57BL/6 (B6; Ly-5.2) recipients. Since Ly-5 is a leukocyte common antigen expressed on all hematopoietic lineages, the origin (donor versus host) of

20   myeloid and lymphoid cells can be followed over time and thus pluripotent HSC engraftment can be evaluated. Ly-5 alleles have been shown not to elicit alloresistance or graft rejection (Sykes, M. et al. (1989) J. Immunol. 143:3503), so engraftment can be evaluated in the absence of alloreactivity. Engraftment was examined following various doses of WBI. Additionally, the time period ("window")

25   for engraftment following WBI was examined.

The following methodology was used in this example:

Animals: Female C57BL/6NCR (B6;h-2^b, Ly-5.2) and Ly-5 congenic

30   B6.Ly-5.2 (Ly-5.1) mice were obtained from the Frederick Cancer Research Facility, Frederick, MD. Ly-5 alleles are described according to the nomenclature of Morse et al. (Immunogenetics (1987) 25:71). All mice were housed in sterilized microisolator cages, in which they received autoclaved food and autoclaved acidified drinking water. Recipients were age-matched and were used at 12-16 weeks of age.

35

BMT: BMT was performed as previously described (Sykes, M. et al. (1993) J. Immunol. 150:197). Briefly, recipient B6 mice were irradiated with various doses (0.5-9.5 Gy) (^{137} Cs source, approximately 1.0 Gy/min) and reconstituted with T cell-depleted (TCD) Ly-5.1 BMC (1.5 x 10^7 unless indicated otherwise), obtained

from the tibiae and femora of sex-matched B6.Ly-5.2 donors aged 6-14 weeks. T cell-
depletion was performed as described (Morse et al. (1987) Immunogenetics 25:71)
using anti-CD4 (Dialynas, D.P. et al. (1983) J. Immunol. 131:2445) and CD8
(Sarmiento, M. (1980) J. Immunol. 125:2665) mAb and C'.

Cell counts: Heparinized peripheral blood was analyzed on an
Automated Cell Counter (System 9000; Serono-Baker Diagnostics, Inc., Allentown,
PA).

Phenotyping of chimeras: Phenotyping was performed at various times
beginning 2 weeks following BMT. Animals were tail bled and white blood cells
(WBC) were prepared by hypotonic shock. Suspensions of spleen cells, thymocytes,
BMC and bone marrow colonies were also analyzed. Staining with both donor-
specific and recipient-specific mAb was performed on each chimera and control
animal. Cells were incubated with 20 μl undiluted culture supernatant of A20-1.7
(anti-Ly-5.1 mAb; mouse IgG2a) or 104-2.1 (anti Ly-5.2 mAb; mouse IgG2a) for 30
minutes at 4°C, then washed twice. In order to block non-specific FcγR binding of
labeled antibodies, 10 μl undiluted culture supernatant of 2.4G2 (rat anti-mouse FcγR
mAb) was added to the first incubation. Cell-bound mAbs were detected with
fluorescein isothiothianate (FITC)-conjugated rat anti-mouse IgG2a mAb (Zymed
Laboratories, Inc., Mundelein, IL), which was incubated for 30 minutes at 4°C,
followed by two washes and analysis on a FACScan (Becton Dickinson, Mountain
View, CA). In all experiments, the percentage of cells staining with each mAb was
determined from one-color fluorescence histograms and comparison with those
obtained from normal donor and host-type animals, which were used as positive and
negative controls. The percentage of cells considered positive after staining with a
mAb was determined using a cutoff chosen as the fluorescence level at the beginning
of the positive peak for the positive control strain, and by subtracting the percentage of
cells stained with an irrelevant mAb (non-reactive IgG2a mAb HOPC1 plus FITC-
conjugated anti-mouse IgG2a mAb). The relative percent staining of a chimera with
mAb was calculated using the formula:
100% x (net chimera percent positive) - (net negative control percent positive)/(net
positive control percent positive) - (net negative control percent positive) where "net"
percent positive refers to the percentage obtained after subtraction of staining with
HOPC1, and positive and negative controls were cells from appropriate normal
Ly5.1$^+$ and Ly5.2$^+$ mice. For test cell populations in which staining with an anti-Ly5
mAb was less than that of the negative control, and the calculated percent chimerism
was therefore less than 0, the values are reported as 0. Using this method of
calculation, less than 0.1% contaminating Ly5.1$^+$ cells could be detected in artificial

Ly5.2(99.9 %)/Ly5.1(0.1%) mixtures. However, a visible positive peak was not detectable in artificial mixtures containing 0.1% or fewer Ly5.1$^+$ cells, but was visible with 1% contaminating Ly5.1$^+$ cells (data not shown). All hematopoietic lineages stained strongly with anti-Ly-5-mAb[10]. By using forward and 90° light scatter (FSC and SSC, respectively) dot plots, lymphocyte (FSC- and SSC-low population), granulocyte (SSC-high population), and monocyte (FSC-high but SSC-low population) populations were gated, and chimerism was determined separately for each population. All SSC-high cells in the granulocyte gate stained with FITC-conjugated anti-mouse granulocyte mAb (Gr-1) (data not shown). Dead cells were excluded by gating out low FSC/high propidium iodide-retaining cells.

Colony-forming unit (CFU) assay: Thirty three thousand BMC were cultured in 1 ml of Iscove's 2.3% methylcellulose (HCC-410; Terry Fox Laboratory, British Columbia, Canada) medium, supplemented with 5% mouse IL-3-containing supernatant (WEHI-3 supernatant, Lot No. 906852; Beckton Dickinson, Bedford, MA), 30% FCS (HyClone, Logan, UT), 5x10^5 M 2-ME, 15% Iscove's modified Dulbecco's medium (Mediatech, Washington, D.C.), and antibiotics (50 U/ml penicillin and 50 μl/mh streptomycin) in a culture flask (Cat. #171099, Nunc, Inc., Naperville, IL). Eight days later, colonies were enumerated, and were then harvested and stained for phenotyping by FCM, as described above.

Statistics: Statistical analysis was performed using the Student t-test. A P value of less than 0.05 was considered to be statistically significant.

Kinetics of hematopoietic recovery in irradiated B6 mice reconstituted with 1.5 x 10^7 Ly-5.1 BMC: In order to assess the degree of myeloablation induced by various doses of WBI, WBC and platelet counts and hemoglobin concentrations were measured at various times following irradiation and BMT. Control Values obtained from untreated B6 mice (n=4) were: WBC 7.6 ±1.5 [5.5-8.6; mean ±standard deviation (SD)] x 10^3/mm^3; hemoglobin 14.8 ± 1.1(13.1-15.5) g/dl; and platelets 13.3 ± 1.4 (12.0 - 15.2) x 10^5/mm^3. As shown in Figure 1a, WBC decreased in proportion to the WBI dose administered. In B6 mice irradiated with 0.5 Gy and injected with Ly-5.1 BMC, WBC numbers did not decrease, but increased above normal from days 14 to 29, then gradually decreased to normal levels by day 42. In mice treated with 1.5 Gy WBI with or without BMT, WBC counts decreased to an average of 3.3 x 10^3/mm^3 by day 2, then gradually increased until day 29. Only animals that received BMT showed an "overshoot" in WBC counts at day 29. In animals irradiated with 3.0 Gy and injected with Ly-5.1 BMC, WBC counts decreased more profoundly to a mean of 2.4 x 10^3/mm^3 by day 4, then gradually recovered to normal levels by day 14. Similar

kinetics were observed in 3 Gy irradiated B6 mice not receiving BMT. Hemoglobin concentrations decreased to an average of 10.6 g/dl by day 4, then gradually recovered to normal levels by day 29 in 3 Gy irradiated B6 mice receiving Ly-5.1 BMC. No change in hemoglobin concentrations was observed in the other groups.

Platelet numbers showed an increase above normal at day 4 that was more marked with increasing irradiation doses. Platelet counts decreased to normal levels by day 7.

3 Gy WBI is required for establishment of stable multilineage hemopoietic chimerism: To examine the kinetics of mixed chimerism in B6 (Ly-5.2$^+$) mice irradiated and reconstituted with congenic Ly-5.1$^+$ BMC, FCM analysis was performed. Since all leukocytes express Ly-5, the Ly-5 alleles expressed on peripheral WBC were analyzed using anti-Ly-5.1 (A20-1.7) and anti-Ly-5.2 (104-2.1) mAbs developed with FITC conjugated anti-mouse IgG2a mAb.

In WBC of unirradiated B6 recipients (n=4) injected with congenic Ly-5.1 BMC, a low percentage of Ly-5.1$^+$cells ($\leq$4%) was detected at 2 and 6 weeks after BMT, but Ly-5.1$^+$ cells were no longer detectable by 20 weeks following BMT. Similar levels of chimerism were observed for lymphocytes, granulocytes, and monocytes. Similar results were obtained in B6 mice (n=5) injected with Ly-5.1 BMC following irradiation with 0.5 Gy WBI. Although initial chimerism was slightly greater than that observed in unirradiated recipients, Ly-5.1$^+$ cells became undetectable by 20 weeks following BMT.

A different result was obtained in B6 mice irradiated with 1.5 Gy and injected with Ly-5.1 BMC. In 2 or 5 recipients, Ly-5.1$^+$ cells increased to 54 and 66% of WBC by 12 weeks after WBI and remained constant for the entire follow-up period of 30 weeks. Similar levels of Ly-5.1 reconstitution were observed for lymphocytes, granulocytes and monocytes of each animal. In the remaining 3 mice, however, Ly-5.1$^+$ WBC did not increase between 2 and 6 weeks post-BMT, and began to decrease at 12 weeks post-BMT. By 30 weeks, donor cells were undetectable in one of these three mice. Although Ly-5.1$^+$ lymphocytes remained detectable in all three mice for at least 20 weeks, granulocyte chimerism was no longer detectable by 6 weeks following BMT.

In contrast to these results, stable and high levels of donor reconstitution were observed in all of 5 B6 mice receiving Ly-5.1 BMC following irradiation with 3 Gy WBI. Donor reconstitution of all lineages increased between 2 and 12 weeks post-BMT, and remained constant thereafter.

Engraftment in recipients of all irradiation doses is summarized in Table 1. There was no statistically significant difference in the level of WBC chimerism between Group 1 and 2. In Groups 2, 3, and 4, however, the level of donor reconstitution at 2 weeks after BMT and the stability of chimerism correlated with

WBI dose. These results show that between 1.5 and 3 Gy WBI is required to permit stable, multilineage reconstitution with injected syngeneic marrow in all recipients.

Chimerism in WBC, spleen cells, thymocytes, BMC, and BM progenitors of B6 recipients of Ly-5.1 BMC 34 to 43 weeks earlier: To evaluate

5   chimerism in other lymphohematopoietic tissues, recipient B6 mice were sacrificed 34-43 weeks after BMT, and peripheral WBC, spleen cells, thymocytes and BMC were harvested and analyzed. At the same time, CFU assays were performed to assess chimerism at the BM progenitor cell level. Eight days after plating, colonies were counted, and all cells in a culture plate were harvested and stained. The number of

10   colonies obtained in the CFU assay was similar for all experimental groups.

Chimerism was not detectable in WBC, spleen cells, thymocytes, BMC, or BM progenitors of any of six B6 mice injected with Ly-5.1 BMC with or without 0.5 Gy WBI.

In the group of Ly-5.2 mice receiving 1.5 Gy WBI and Ly-5.1 BMC,

15   variable results were obtained, as had been observed for WBC. The level of donor reconstitution of peripheral granulocytes, spleen cells, thymocytes, BMC, and BM progenitors generally correlated with the total percentage of donor cells in peripheral WBC. In one mouse, about 70-80% of WBC, granulocytes, spleen cells, thymocytes, BMC, and BM progenitors were donor-derived. Chimerism was undetectable in any

20   tissues of another mouse. In two other mice, chimerism was not detectable for granulocytes, BMC, or BM progenitors. However, a low level of chimerism was clearly detected for WBC and spleen cells, or for thymocytes only.

High levels of donor repopulation were observed for all cell populations of Ly-5.2 mice irradiated with 3 Gy and injected with Ly-5.1$^+$ BMC.

25   Lack of evidence for quiescent Ly-5.1 hematopoietic stem cells in BMC of Ly-5.2 mice that had received Ly-5.1 BMC following conditioning with 0.5 Gy WBI: In order to further evaluate the possible presence of low numbers of Ly-5.1 hematopoietic stem cells in recipient Ly-5.2 mice in which chimerism had been initially detected but later disappeared, we transformed TCD BMC of these mice to

30   secondary supralethally irradiated (9.5 Gy) Ly-5.2 recipients. Two BMC doses were used (10^5 and 10^6). It was expected that recipients of the higher dose of cells might not show levels of chimerism that were different from those in the primary recipients. In contrast, recipients of low numbers of marked stem cells in competitive repopulation assays often show levels of repopulation by the marked population that

35   diverge markedly from the percentage of such cells in the reconstituting inoculum[11-13]. Thus, if a group of animals were reconstituted with low numbers of HSC consisting mostly of Ly-5.2$^+$ cells plus a low (undetectable by FACS) percentage of Ly-5.1$^+$ cells, some secondary recipients might show Ly-5.1$^+$ detectable levels of Ly-5.1 chimerism. Results obtained in recipients of artificial BMC mixtures (95 % Ly-

5.2^+, 5% Ly-5.1$^+$) confirmed this prediction, as long-term chimerism was close to 5% in all recipients of 10^6 BMC (Group 2, Table II), but ranged from 0-28% in recipients of smaller numbers (10^6 of the same cell mixture (Group 1, table II).

5   As shown for Groups 3-6 in Table II, Ly-5.1$^+$ cells were not detected in WBC of Ly-5.2 mice irradiated with 9.5 Gy and injected with either 10^{5}or 10^6 TCD BMC from Ly-5.2 mice (mouse numbers 2544 and 2548) which had been irradiated with 0.5 Gy and injected with Ly-5.1$^+$ BMC 43 weeks earlier.

"Space" for BM engraftment persists for at least 7 days following 3 Gy WBI: We investigated the relationship between timing of 3 Gy WBI and the ability of
10   Ly-5.1$^+$ BMC to graft in Ly-5.2 recipients. Irradiated Ly-5.2 mice received Ly-5.1 BMC at various intervals from 0-21 days following irradiation (Table III). In all animals irradiated on day 0 and receiving Ly-.5.1 BMC on day 0, 4, or 7, high levels of donor-type repopulation were observed, and remained stable for at least 20 weeks (Table III, groups 1-3). In contrast, mixed chimerism was induced in only 3 of 4 and 2
15   of 4 recipients of Ly-5.1 BMC on day 14 and 21, respectively (Table III, groups 4 and 5). In B6 mice in which Ly-5.1$^+$ cells were initially detected only in the lymphoid but not in the granulocyte lineage, chimerism disappeared completely by 20 weeks following WBI.

A decline in WBC counts was observed in 3 Gy irradiated mice 2-7 days
20   after irradiation, but these counts recovered to normal by day 14 (shown in Figure 1A for recipients of Ly-5.1 BMC). In recipients of 1.5 Gy WBI with or without BMT, a reduction in WBC counts was also observed in this period (Figure 1A), whereas no significant decline was observed in recipients of lower doses. Thus, these data indicate that reductions in WBC counts can serve as a marker for the presence of
25   "space" for HSC engraftment. However, WBC counts and space for engraftment do not correlate perfectly since WBC counts recover to normal at a time (e.g., about 14 days after WBI) when space for HSC engraftment is still present in many animals.

WO 95/21527

PCT/US94/01616

24

Table I

Kinetics of Chimerism in WBC of B6 (Ly-5.2) Mice Irradiated and Injected with 1.5 x 10^7 Ly-5.1 BMC

Group[a]	WBI Dose (Gy)	No. of Mice	% Donor (Ly5.1) WBC[b] (Mean + SD) (No. of Mice with Mixed Chimerism				% Recipient (Ly-5.2) WBC (Mean ± SD)			
			2 wks	6 wks	12 wks	30 wks	2 wks	6 wks	12 wks	30wks
1	0	4	2/0 ± 0.8 (4/4)	1.5 ± 1.7 (2/4)	0.5 ± 0.8 (1/4)	0.2 ± 0.1 (0/4)	95.9 ± 2.7	96.6 ± 1.6	98.2 ± 1.3	99.4 ± 0.7
2	0.5	5	2.4 ± 0.5[c] (5/5)	2.4 ± 0.5[c] (4/5)	1.3 ± 1.2[c] (1/5)	0.0 ± 0.0[c] (0/5)	95.0 ± 1.3[c]	96.8 ± 0.6[c]	97.6 ± 2.2[c]	99.6 ± 0.4[c]
3	1.5	5	10.4 ± 3.2[d] (5/5)	28.3 ± 20.4[d] (5/5)	29.0 ± 28.7[d] (5/5)	25.1 ± 32.4[f] (3/5)	85.2 ± 4.4[d]	63.4 ± 16.9[d]	66.5 ± 24.7[d]	70.9 ± 35.3[f]
4	3	5	31.3 ± 8.0[c] (5/5)	72.1 ± 5.0[c] (5/5)	82.6 ± 0.7[a] (5/5)	86.0 ± 3.2[c] (5/5)	62.2 ± 5.4[c]	26.0 ± 5.4[c]	18.2 ± 1.5[c]	8.0 ± 1.8[c]

a) Recipient B6 mice were irradiated with various dose (0-3 Gy) and injected with 1.5 x 10^+ TCD BMC from B6.Ly-5.2 mice.

b) Chimerism was tested by FCM at various intervals after BMT.

SUBSTITUTE SHEET (RULE 26)

c) Not significant compared with Group 1.

d) $P < 0.01$ compared with Group 2.

e) $P < 0.01$ compared with Group 3.

f) Not significant compared with Group 2.

Table II

Ly5.1$^+$ WBC are Not Detectable in Secondary, 9.5 Gy-Irradiated Recipients of TCD BMC from Ly5.2$^+$ Recipients of 0.5 Gy WBI and Ly5.1$^+$ BMC 43 Weeks Earlier

5

	TCD BMC from		Fraction of Chimeric Animals[c] (% Ly5.1$^+$ Cells/Mean±SD)	
Group[a]	Donor (Pretreatment[b])	Cell Dose (x10^5)	2 wks	8 wks
1	95 % Ly-5.2 BMC + 5 % Ly-5.1 BMC (Untreated)	1	10/10 (1.2-13.5 / 5.0±3.4)	8/10 (0-28.1 / 5.8±9.2)
2	95 % Ly-5.2 BMC + 5 % Ly-5.1 BMC (Untreated)	10	10/10 (3.6-6.1 / 4.7±1.0	10/10 (2.4-6.3/ 4.9±1.2)
3	2544 (0.5 Gy + Ly5.1$^+$ BMC)	1	0/10 (0/ 0/0^d±0.0)	0/10 (0/ 0/0^d±0.0)
4	2544 (0.5 Gy + Ly5.1$^+$ BMC)	10	0/10 (0/ 0/0^d±0.0)	0/10 (0/ 0/0^d±0.0)
5	2548 (0.5 Gy + Ly5.1$^+$ BMC)	1	0/10 (0/ 0/0^d±0.0)	0/10 (0/ 0/0^d±0.0)
6	2548 (0.5 Gy + Ly5.1$^+$ BMC)	10	0/10 (0/ 0/0^d±0.0)	0/10 (0/ 0/0^d±0.0)

a) Recipient B6 mice were irradiated with supralethal dose of 9.5 Gy and reconstituted with 1 or 10 x 10^5 TCD BMC.

SUBSTITUTE SHEET (RULE 26)

    b) Donor animal was untreated or Mouse number 2544 and 2548 were treated with 0.5 Gy WBI followed by injection of 1.5×10^7 TCD BMC from untreated B6.Ly-5.2 mice 43 weeks earlier.

    c) See footnote[b] of Table I.

5   d) All values for staining with anti-Ly-5.1 mAb followed by FITC-conjugated anti-mouse IgG2a mAb were less than or equal to 0 after subtraction of staining with control mAb (HOPCI-1 followed by FITC-conjugated anti-mouse IgG2a).

Table III

"Space" for BM Engraftment in All Recipients Persists for at least 7

days

following 3 Gy WBI

5

Group[a]	Injection of Ly5.1[+]	No. of	% Donor (Ly5.1) Cellsb (Mean ±SD) (No. of Mice with WBC Mixed Chimerism) [No. of Mice with Granulocyte Mixed Chimerism			
	BMC on Day X	Mice	3 wks (after WBI)	6 wks	12 wks	20 wks
1	0	4	22.6 ± 3.1 (4/4) [4/4]	38.7 ± 6.1 (4/4) [4/4]	62.7 ± 2.1 (4/4) [4/4]	69.3 ± 1.7 (4/4) [4/4]
2	4	4	17.7 ± 7.3 (4/4) [4/4]	48.6 ± 5.0 (4/4) [4/4]	68.5 ± 4.6 (4/4) [4/4]	81.1 ± 4.0 (4/4) [4/4]
3	7	4	14.1 ± 4.5 (4/4) [4/4]	32.9 ± 9.3 (4/4) [4/4]	55.9 ± 7.8 (4/4) [4/4]	69.7 ± 7.5 (4/4) [4/4]
4	14	4	1.3 ± 0.6 (3/4) [3/4]	15.6 ± 10.4 (4/4) [3/4]	23.1 ± 14.9 (3/4) [3/4]	32.5 ± 30.0 (3/4) [3/4]
5	21	4	-	9.7 ± 7.8 (4/4) [2/4]	23.1 ± 21.6 (3/4) [2/4]	30.5 ± 34.9 (2/4) [2/4]

a) Recipient B6 mice were irradiated with 3 Gy on day 0 and injected with 1.5 x 10^{7} TCD BMC

b) See footnote[b] of Table I.

5

Other Embodiments

Stromal tissue introduced prior to hematopoietic cell transplant, e.g., BMT, may be varied by: (1) administering the fetal liver and thymus tissue as a fluid cell suspension; (2) administering fetal liver or thymus stromal tissue but not both; (3) placing a stromal implant into other encapsulated, well-vascularized sites, or (4) using adult thymus or fetal spleen as a source of stromal tissue.

The methods described herein for inducing tolerance to an allogeneic antigen or allogeneic graft can be used where, as between the donor and recipient, there is any degree of mismatch at MHC loci or other loci which influence graft rejection. Preferably, there is a mismatch at at least one MHC locus or at at least one other locus that mediates recognition and rejection, e.g., a minor antigen locus. With respect to class I and class II MHC loci, the donor and recipient can be: matched at class I and mismatched at class II; mismatched at class I and matched at class II; mismatched at class I and mismatched at class II; matched at class I, matched at class II. In any of these combinations other loci which control recognition and rejection, e.g., minor antigen loci, can be matched or mismatched. As stated above, it is preferable that there is mismatch at least one locus. Mismatched at MHC class I means mismatched for one or more MHC class I loci, e.g., in the case of humans, mismatched at one or more of HLA-A, HLA-B, or HLA-C, or in the case of swine, mismatch at one or more SLA class I loci, e.g., the swine A or B loci. Mismatched at MHC class II means mismatched at one or more MHC class II loci, e.g., in the case of humans, mismatched at one or more of a DP α, a DPβ, a DQ α, a DQ β, a DR α, or a DR β, or in the case of swine, mismatch at one or SLA class II loci, e.g., mismatch at DQ α or β, or DR α or β.

The methods described herein for inducing tolerance to an allogeneic antigen or allogeneic graft can be used where, as between the donor and recipient, there is any degree of reactivity in a mixed lymphocyte assay, e.g., wherein there is no, low, intermediate, or high mixed lymphocyte reactivity between the donor and the recipient. In preferred embodiments mixed lymphocyte reactivity is used to define mismatch for class II, and the invention includes methods for performing allogenic grafts between individuals with any degree of mismatch at class II as defined by a mixed lymphocyte assay. Serological tests can be used to determine mismatch at class I or II loci and the invention includes methods for performing allogenic grafts between individuals with any degree of mismatch at class I and or II as measured with

serological methods. In a preferred embodiment, the invention features methods for performing allogeneic grafts between individuals which, as determined by serological and or mixed lymphocyte reactivity assay, are mismatched at both class I and class II.

The methods of the invention are particularly useful for replacing a

5   tissue or organ afflicted with a neoplastic disorder, particularly a disorder which is resistant to normal modes of therapy, e.g., chemotherapy or radiation therapy. Methods of the invention can be used for inducing tolerance to a graft, e.g., an allograft, e.g., an allograft from a donor which is mismatched at one or more class I loci, at one or more class II loci, or at one or more loci at each of class I and class II.

10   In preferred embodiments: the graft includes tissue from the digestive tract or gut, e.g., tissue from the stomach, or bowel tissue, e.g., small intestine, large intestine, or colon; the graft replaces a portion of the recipient's digestive system e.g., all or part of any of the digestive tract or gut, e.g., the stomach, bowel, e.g., small intestine, large intestine, or colon.

15   Tolerance, as used herein, refers not only to complete immunologic tolerance to an antigen, but to partial immunologic tolerance, i.e., a degree of tolerance to an antigen which is greater than what would be seen if a method of the invention were not employed.

As is discussed herein, it is often desirable to expose a graft recipient to

20   irradiation in order to promote the development of mixed chimerism. The inventor has discovered that it is possible to induce mixed chimerism with less radiation toxicity by fractionating the radiation dose, i.e., by delivering the radiation in two or more exposures or sessions. Accordingly, in any method of the invention calling for the irradiation of a recipient, e.g., a primate, e.g., a human, recipient, of a xenograft or

25   allograft, the radiation can either be delivered in a single exposure, or more preferably, can be fractionated into two or more exposures or sessions. The sum of the fractionated dosages is preferably equal, e.g., in rads or Gy, to the radiation dosage which can result in mixed chimerism when given in a single exposure. The fractions are preferably approximately equal in dosage. For example, a single dose of 700 rads

30   can be replaced with, e.g., two fractions of 350 rads, or seven fractions of 100 rads. Hyperfractionation of the radiation dose can also be used in methods of the invention. The fractions can be delivered on the same day, or can be separated by intervals of one, two, three, four, five, or more days. Whole body irradiation, thymic irradiation, or both, can be fractionated.

35   The inventor has also discovered that much or all of the preparative regimen can be delivered or administered to a recipient, e.g., an allograft or xenograft recipient, within a few days, preferably within 72, 48, or 24 hours, of transplantation of tolerizing stem cells and/or the graft. This is particularly useful in the case of humans receiving grafts from cadavers. Accordingly, in any of the methods of the

invention calling for the administration of treatments prior to the transplant of stem cells and/or a graft, e.g., treatments to inactivate or deplete host antibodies, treatments to inactivate host T cells or NK cells, or irradiation, the treatment(s) can be administered, within a few days, preferably within 72, 48, or 24 hours, of
5   transplantation of the stem cells and/or the graft. In particular, primate, e.g., human, recipients of allografts can be given any or all of treatments to inactivate or deplete host antibodies, treatments to inactivate host T cells or NK cells, or irradiation, within a few days, preferably within 72, 48, or 24 hours, of transplantation of stem cells and/or the graft. For example, treatment to deplete recipient T cells and/or NK cells,
10   e.g., administration of ATG, can be given on day -2, -1, and 0, and WBI, thymic irradiation, and stem cell, e.g., bone marrow stem cells, administered on day 0. (The graft, e.g., a renal allograft, is transplanted on day 0).

As described herein, it has been discovered that there is a permissible
15   time period ("window") for hematopoietic stem cell engraftment following creation of space for the donor hematopoietic stem cell (e.g., by whole body irradiation) in a recipient. It has further been discovered that space created for hematopoietic stem cell engraftment can be monitored over time by monitoring white blood cell levels in a recipient. Accordingly, in any method which involves hematopoietic stem cell
20   transplantation, and thus also requires creation of space in a recipient for donor hematopoietic stem cell, transplantion can be performed during the permissible window for engraftment following creation of space for the hematopoietic stem cell, as provided by the invention. Likewise, in any method in which space is created for exogenously administered hematopoietic stem cells, white blood cell levels can be
25   monitored to monitor space for the donor hematopoietic stem cells (i.e., to assess the permissible window for engraftment). Examples of procedures involving hematopoietic stem cell transplantation include 1) conditioning of a recipient for an allo- or xenograft, as described herein, in which hematopoietic stem cell transplantation is performed in conjunction with transplantation of another allo- or
30   xenograft; 2) treatment of various hematopoietic disorders, including leukemias, lymphomas and other hematopoietic malignancies and genetic hematopoietic disorders (e.g., adenosine deaminase deficiency, bare lymphocyte syndrome and other congenital immunodeficiency diseases) in which hematopoietic stem cell transplantation is performed therapeutically; and 3) transplantation of genetically
35   modified hematopoietic stem cells (e.g., genetically modified autologous hematopoietic stem cells) to deliver a gene product to a recipient (e.g., as gene therapy).
Furthermore, the findings of the current invention regarding a permissible time window for hematopoietic stem cell engraftment, and the ability to

monitor this window by monitoring white blood cell levels in the recipient, can be applied to any type of bone marrow transplantation situation, e.g., autologous, syngeneic, allogeneic or xenogeneic transplantation. For example, syngeneic bone marrow transplantation is the preferred method for gene therapy using hematopoietic

5   stem cells (i.e., an individuals own stem cells are isolated, genetically modified and reintroduced into the individual) whereas allogeneic or xenogeneic bone marrow transplantation is necessary for conditioning of a recipient of an allograft or xenograft.

The discovery of a permissible time window for engraftment following creation of space for donor bone marrow (e.g., following whole body irradiation) in a

10   recipeint is useful for allowing more flexibility in the preparative regimen of a hematopoietic stem cell recipient. Often a BMT recipient undergoes several preparative treatments which can be stressful and debilitating to the recipient. The ability to allow for some "recovery time" for the recipient following whole body irradiation (or other procudure to create space) prior to administering bone marrow

15   cells is thus advantageous. Furthermore, the ability to monitor space for donor hematopoietic stem cell within a recipient (e.g., by white blood cell counts) allows for selection of a suitable time for administration of donor bone marrow and ensures that the optimal window for engraftment is not missed.

It has further been discovered that thymic irradiation of a recipient can

20   be replaced by treatment of the recipient with (mature and immature) T cell ablative antibodies. Accordingly, in any method calling for thymic irradiation in order to deplete mature and immature T cells within the recipient, administration of T cell ablative antibodies can be substituted for thymic irradiation. Procedures involving thymic irradiation can include preparative treatment of a recipient for an allograft in

25   conjunction with hematopoietic stem cell (as described herein) or preparative treatment of a recipient of a hematopoietic stem cell transplant alone (e.g., as therapy for a hematopoietic disorder or for gene therapy purposes, as discussed above). The ability to replace thymic irradiation with administration of T cell ablative antibodies as a preparative step for a transplant recipient reduces the amount of irradiation to which

30   a recipient need be exposed. This is an advantageous improvement considering the possible unwanted side effects (e.g., mutations, toxicity) which can occur from excessive irradiation.

Other embodiments are within the following claims.

What is claimed is:

1. A method of evaluating a tretment for the ability to create hematopoietic space in a recipient comprising administering a myelosuppressive treatment to said recipient, determining the level of white blood cells in the recipient after said treatment and comparing the post-treatment level with the pre-treatment
5   level of white blood cells, a depression in the level of white blood cells being indicative of the presence or induction of hematopoietic space.

2. The method of claim 2, wherein said myelosuppressive treatment comprises irradiation of said recipient.
10

3. A method of promoting the engraftment of exogenously administered hematopoietic stem cells in a recipient comprising administering myelosuppressive treatment to the recipient to create hematopoietic space, determining the white blood cell level of the recipient, admininistering said hematopoietc cells to said recipient
15   during a period in which the white blood cell level is depressed.

4. The method of claim 3, wherein said myelosuppressive treatment comprises irradiation of said recipient.

20   5. The method of claim 3, further comprising implanting in the recipient, a graft from a donor.

retro xeno

25   6. A method of inducing tolerance in a recipient mammal of a first species to a tissue obtained from a mammal of a second species, which tissue expresses an MHC antigen, said method comprising inserting DNA encocling an MHC antigen of said second species into a hematopoietic stem cell from said recipient mammal, administering a hematopoietic space inducing treatment to said recipient,
30   monitoring the white cell level of said recipient after said administration of hematopoietic space inducing treatment, administering said stem cells to said recipient while said white cell level is depressed, and allowing said MHC antigen encoding DNA to be expressed in the recipient.

35   7. The method of claim 6, wherein said hematopoietic space inducing treatment comprises irradiation of said recipient.

8. The method of claim 6, wherein said DNA is inserted into said cell by a retrovirus.

9. A method of inducing tolerance in a recipient mammal to a tissue obtained from a donor mammal of the same species, which tissue expresses an MHC antigen, said method comprising inserting DNA encoding an MHC antigen of said

5   donor into a hematopoietic stem cell from said recipient, administering a hematopoietic space inducing treatment to said recipient, monitoring the white cell level of said recipient after said administration of hematopoietic space inducing treatment, administering said stem cells to said recipient while said white cell level is depressed, and allowing said MHC antigen encoding DNA to be expressed in the

10   recipient.

10. The method of claim 9, wherein said hematopoietic space inducing treatment comprises irradiation of said recipient.

15   11. The method of claim 9, wherein said DNA is inserted into said cell by a retrovirus.

12. A method of inducing tolerance in a recipient mammal of a first species to a graft obtained from a mammal of a second species, said method

20   comprising administering a hematopoietic space inducing treatment to said recipient, monitoring the white cell level of said recipient after said administration of hematopoietic space inducing treatment, administering donor hematopoietc stem cells to said recipient while said white cell level is depressed, and implanting said graft in said recipient.
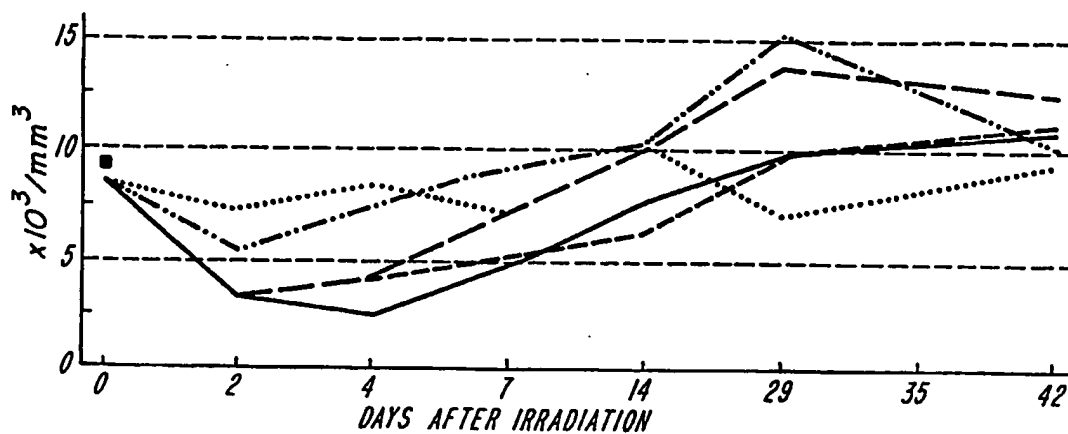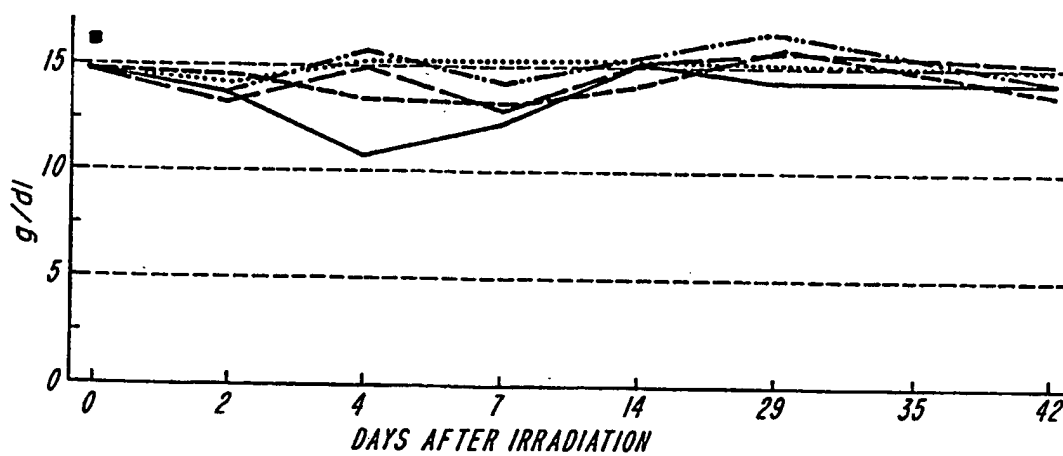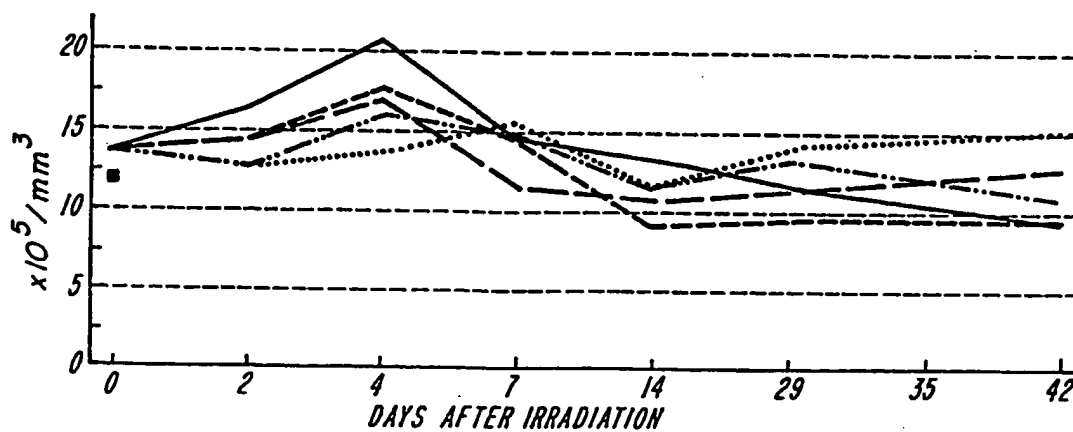
25

13. The method of claim 12, wherein said hematopoietic space inducing treatment comprises irradiation of said recipient.

14. The method of claim 12, further comprising, introducing into the

30   recipient mammal donor species-specific stromal tissue.

15. A method of inducing tolerance in a recipient mammal to a graft obtained from a donor of the same species, said method comprising said method comprising administering a hematopoietic space inducing treatment to said recipient,

35   monitoring the white cell level of said recipient after said administration of hematopoietic space inducing treatment, administering donor hematopoietc stem cells to said recipient while said white cell level is depressed, and implanting said graft in said recipient.

16. The method of claim 15, wherein said hematopoietic space inducing treatment comprises irradiation of said recipient.

FIG. 1A

FIG. 1B

FIG. 1C

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A01N 63/00; A61K 37/00, 49/00; G01N 1/00, 31/00, 33/15, 33/48;

US CL :424/2, 9, 93R, 93A, 93B

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/2, 9, 93R, 93A, 93B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TRANSPLANTATION, Volume 54, Number 3, Issued September 1992, B.R. Rosengard et al., "INDUCTION OF SPECIFIC TOLERANCE TO CLASS I-DISPARATE RENAL ALLOGRAFTS IN MINIATURE SWINE WITH CYCLOSPORINE", pages 490-497, see entire document.	1 and 2
Y	TRANSPLANTATION PROCEEDINGS, Volume 21, Number 1, issued February 1989, J.C. Madsen et al., "Induction of Immunological Unresponsiveness Using Recipient Cells Transfected with Class I or Class II MHC Genes", page 477, see entire document.	1 and 2

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 MAY 1994	2 3 MAY 1994

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer Brian R. Stanton
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)

INTERNATIONAL SEARCH REPORT

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TRENDS IN GENETICS, Volume 2, issued June 1986, J.E. Dick et al., "Genetic manipulation of hematopoietic stem cells with retrovirus vectors", pages 165-170, see entire document.	1 and 2
Y	HUMAN IMMUNOLOGY, Volume 28, issued 1990, D.H. Sachs et al., "Immunology of Xenograft Rejection", pages 245-251, see entire document.	1 and 2
X	THE JOURNAL OF EXPERIMENTAL MEDICINE, Volume 169, Issued February 1989, Y. Sharabi et al., "MIXED CHIMERISM AND PERMANENT SPECIFIC TRANSPLANTATION TOLERANCE INDUCED BY A NONLETHAL PREPARATIVE REGIMEN", pages 493-502, see entire document.	1 and 2

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, Medline, Biosis, CA, EMBASE
Search Terms: Hematopoi?; graft?; irradia?; lethal?; MHC; histocompat?; retrovir?; stroma?; assay#; monitor?; sachs?/au; sykes?/au

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

The instantly claimed invention lacks unity of invention and comprises the following groups of inventions:

I.   Claims 1 and 2, drawn to treatment evaluation methods, classified in Class 424, subclass 9.

II.   Claims 3-5 and 12-15, drawn to transplantation methods using non-recombinant cells, classified in Class 424, subclass 93R.

III.   Claims 6-11, drawn to transplantation methods using recombinant cells, classified in Class 424, subclass 93B.

The inventions are distinct, one from the other for the following reasons:

> The invention of Group I is distinct from either of the inventions of Groups II or III because the claimed treatment evaluation methods do not require any cellular transplantation and therefore no search or consideration of such is required.

> The inventions of Groups II and III are distinct, one from the other because the methods of Group III require the use of recombinant cells which necessitates consideration and search of transfection methods, genes, vectors, etc. and such considerations are not required for analysis of the invention of Group II.

Therefore, for the reasons elaborated above, the claimed methods lack any special technical feature within the meaning of PCT Rule 13.2.

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 1-26 are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compound/ composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9309234	13-05-93	AU-A-	3130093	07-06-93
		CA-A-	2124858	13-05-93
WO-A-9218615	29-10-92	CA-A-	2107897	10-10-92
		EP-A-	0584184	02-03-94

Form PCT/ISA/210 (patent family annex) (July 1992)